Suppressors of transcriptional transgenic silencing in *Chlamydomonas* are sensitive to DNA-damaging agents and reactivate transposable elements

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In the unicellular green alga *Chlamydomonas reinhardtii*, the epigenetic silencing of transgenes occurs, as in land plants, at both the transcriptional and posttranscriptional levels. In the case of single-copy transgenes, transcriptional silencing takes place without detectable cytosine methylation of the introduced DNA. We have isolated two mutant strains, Mut-9 and Mut-11, that reactivate expression of a transcriptionally silenced single-copy transgene. These suppressors are deficient in the repression of a DNA transposon and a retrotransposon-like element. In addition, the mutants show enhanced sensitivity to DNA-damaging agents, particularly radiomimetic chemicals inducing DNA double-strand breaks. All of these phenotypes are much more prominent in a double mutant strain. These observations suggest that multiple partly redundant epigenetic mechanisms are involved in the repression of transgenes and transposons in eukaryotes, presumably as components of a system that evolved to preserve genomic stability. Our results also raise the possibility of mechanistic connections between epigenetic transcriptional silencing and DNA double-strand break repair.

Epigenetic processes, which result in heritable changes in gene expression without modifications in DNA sequence, play important roles in the control of development as well as in the cellular responses to viruses, viroids, transposable elements, and transgenes (1–5). In plants, fungi, and animals, analyses of transgene expression have revealed a wide range of epigenetic silencing processes and are providing new insights into mechanisms of gene regulation. Depending on the level at which silencing occurs, two types of phenomena have been distinguished: transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) (1, 4–7). In addition, the introduction of double-stranded RNA triggers a process similar to PTGS, called RNA interference, in a variety of protozoa, invertebrate, and vertebrate species (8–10). In *Chlamydomonas reinhardtii*, transgenes are silenced by epigenetic phenomena similar to those in land plants (11–13).

PTGS involves sequence specific degradation of RNAs. Several genes required for RNA interference (RNAi) or related posttranscriptional processes, such as quelling, have been isolated in animal and fungal systems (4, 9, 10, 14–16). Thus far, four genes have been implicated in PTGS in *Arabidopsis thaliana*: *SDE1/SGS2*, encoding an RNA-dependent RNA polymerase; *AGO1*, encoding a protein similar to rabbit eIF2C; *SDE3*, encoding an RNA helicase; and *SGS3* encoding a coiled-coil protein of unknown function (4, 7, 9, 10, 17). In *Chlamydomonas*, we have recently described a DEAH-box RNA helicase that functions in the posttranscriptional silencing of transgenes and transposons (13). Although the molecular mechanism(s) of RNAi/PTGS is not fully understood, recent evidence indicates that double-stranded RNA, generated by alternative pathways, is processed to 21- to 25-nt RNAs by an RNase-III-related protein (4, 7, 10, 15, 16). These small RNAs target the cleavage of homologous transcripts through an RNA-directed ribonuclease, a multisubunit complex named RNA-induced silencing complex in *Drosophila* (4, 7, 10, 15, 16).

TGS involves transcriptional repression. In plants, it is usually associated with cytosine methylation of promoter regions and reduced accessibility to DNase I, suggesting an altered chromatin structure (1, 5, 6, 18). Many transcriptionally silenced transgenes have complex structures, such as arrays of rearranged copies integrated at a single genomic site (5, 6). In a phenomenon resembling paramutation, some of these loci can also silence homologous sequences in trans (5, 6). DNA–DNA interactions have long been postulated to trigger this homology-dependent process (1, 18–20). However, double-stranded RNA derived from promoter regions has recently been implicated in the transcriptional inactivation of homologous sequences in ectopic positions (4, 21–23). These findings also raise the possibility of mechanistic connections between PTGS and TGS (18, 21–24).

Several genes required for TGS of transgenes have been identified in *Arabidopsis*. *DDM1* (Decrease in DNA Methylation) encodes a chromatin-remodeling protein belonging to the SWI2/SNF2 superfamily, which affects both genomic DNA methylation and TGS (25). *MOM1* (Morpheus' Molecule) encodes a nuclear protein that releases TGS without changes in transgene methylation (26). These genes also control some transposable elements (3, 27–29). Histone deacetylases and DNA methyltransferases also play a role in the epigenetic regulation of (trans)gene expression in *Arabidopsis* (2, 22, 24, 30, 31). In *Drosophila melanogaster* and *Caenorhabditis elegans*, the transcriptional silencing of repeated transgenes depends on Polycomb Group (PcG) genes, initially defined by their function in the repression of developmental genes (32, 33). However, the role of plant PcG homologs in TGS is currently unknown (3).

In *Chlamydomonas* and other volvocine algae, as in land plants, silenced multiple-copy transgenes exhibit high levels of DNA methylation (12, 34). In contrast, single-copy transgenes are subject to TGS without detectable cytosine methylation (12). The molecular mechanism(s) of TGS for simple single-copy transgenes has not been examined extensively in higher plants (5, 6, 35, 36). However, some transgenic loci in *Arabidopsis* remain transcriptionally silent despite a drastic reduction in DNA methylation caused by the depletion of methyltransferase 1 (5, 18, 24, 37). Further, in a recent study of silencing of a neomycin phosphotransferase transgene in *Arabidopsis*, single-copy transgenes did not show methylation of a diagnostic *Sac*II promoter site that was partially or completely methylated in all examined multiple-copy lines (36). As previously proposed, these observations suggest that TGS in photosynthetic eukaryotes can also operate through a methylation-independent pathway (12).

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Abbreviations: TGS, transcriptional gene silencing; PTGS, posttranscriptional gene silencing; DSBs, DNA double-strand breaks; TAP, Tris-acetate-phosphate; UV-C, UV light <280 nm.

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To identify the genetic determinants of TGS for single-copy transgenes, we have isolated *Chlamydomonas* mutants deficient in this process. We report here the characterization of two mutant strains, Mut-9 and Mut-11, that reactivate transgenic expression. In addition, the suppressors are defective in the regulation of transposable elements. Interestingly, these *Chlamydomonas* mutants are also very sensitive to DNA-damaging agents causing DNA double-strand breaks (DSBs). Emerging evidence in a variety of eukaryotes suggests that repair of DSBs is associated with chromatin modifications (38, 39). We speculate that the proteins disrupted in Mut-9 and Mut-11 likely function in the formation of a distinct chromatin structure that is required for transcriptional repression and, possibly, DSB repair.

Materials and Methods

Culture Conditions, Strains, and Genetic Screen for Suppressors of Transgenic Silencing. Unless noted otherwise, *C. reinhardtii* cells were grown photoheterotrophically in Tris-acetate-phosphate (TAP) medium (40) as previously described (11, 12). Strain 11-P[300] was generated by transformation of the wild-type strain CC-124 and contains a transcriptionally silenced single copy of the *RbcS2*::*aadA*::*RbcS2* transgene (11, 12). To identify suppressors of transgene silencing, we mutagenized 11-P[300] by transformation with a mutant form of protoporphyrinogen oxidase (*rs-3* gene) (41), conferring resistance to diphenyl ether herbicides. Herbicide-resistant transformants, containing the *rs-3* gene integrated at random into the nuclear genome, were tested for their ability to grow in the presence of spectinomycin as an indication of reactivation of expression of the *aadA* transgene.

Genetic Analyses. We isolated two spectinomycin-resistant mutant strains, Mut-9 and Mut-11. To test whether the insertional mutagen (*rs-3* gene) cosegregated with reactivation of transgenic expression, Mut-9 and Mut-11 were crossed to the wild-type strain of opposite mating type, CC-125, and tetrads were dissected as previously described (40). Meiotic tetrad products of each mutant, containing exclusively the *rs-3* plasmid, were then backcrossed to 11-P[300]. Tetrad products of Mut-9 and Mut-11 were also crossed to each other to generate a double mutant (Mut-9 Mut-11). Expression of the *RbcS2*::*aadA*::*RbcS2* transgene in the tetrad progeny was evaluated by spot tests on medium containing spectinomycin. Five-microliter aliquots of appropriately diluted cells were pipetted onto the plates and incubated as previously described (12). The presence of the transgene and/or the *rs-3* plasmid in the genome was examined by Southern blot analyses.

DNA and RNA Analyses. Total cell DNA was isolated, fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized as previously described (11, 42). Total cell RNA was purified with the RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA), and standard techniques were used for fractionation by formaldehyde–agarose gel electrophoresis, blotting, and hybridization (11, 42). The PhosphorImager System (Molecular Dynamics) was used for quantitation of ^{32}P radioactivity.

Transposon Mobilization Analyses. To test the effect of the mutations on the activity of *Chlamydomonas* transposons, we established parallel subcultures of strains 11-P[300], Mut-9, Mut-11, and Mut-9 Mut-11. Cells were plated at low density to obtain individual colonies. Ten independent colonies from each strain were subcultured by transfer to fresh TAP plates every 2 weeks. After 3 months, we isolated total cell DNA from the subclones and evaluated the mobilization of transposable elements by Southern blot analyses. Genomic DNA was digested with restriction enzymes that cut inside each transposon (conserved site) and in a flanking chromosomal region (polymorphic site depending on the place of insertion) and probed with short DNA sequences that hybridize to the transposon termini.

Growth Rate and Cell Survival on Exposure to DNA-Damaging Agents. Cells were grown photoheterotrophically in TAP medium under continuous light (300 μmol·m^{-2}·s^{-1} photosynthetically active radiation) at 23°C. To determine growth rates, cells in middle logarithmic growth phase were inoculated into fresh TAP medium to a density of 1×10^5 cells/ml. The cells were then cultured under the same conditions and cell densities determined by measuring optical absorbance at 750 nm. For treatment with DNA-damaging agents, cells were grown to logarithmic phase and spread to a density of 500–700 cells per plate. To test for sensitivity to UV light below 280 nm (UV-C), cells spread on minimal HS medium (40) were irradiated with a Stratalinker (Stratagene). After 24 h in the dark, to prevent photoreactivation, the plates were incubated under moderate light (50 μmol·m^{-2}·s^{-1} photosynthetically active radiation) at 23°C for 10–14 days before the surviving colonies were counted. For treatments with bleomycin (Zeocin; Invitrogen) and methyl methanesulfonate (Sigma), cells were spread on TAP plates containing the appropriate concentrations of each genotoxic agent and incubated as described above.

Results

Isolation of *Chlamydomonas* Mutants Defective in Transcriptional Transgene Silencing. To identify genes responsible for epigenetic silencing in *C. reinhardtii*, we carried out random insertional mutagenesis on strain 11-P[300], which contains a transcriptionally silenced single copy of the *RbcS2*::*aadA*::*RbcS2* transgene (12). This transgene consists of the coding sequence of the eubacterial *aadA* gene (conferring spectinomycin resistance) under the control of the 5′ and 3′ regulatory regions of the endogenous *RbcS2* gene (encoding the small subunit of Rubisco) (11). Because *Chlamydomonas* is haploid, nonlethal mutations in genes required for silencing allow reactivation of expression of *aadA* and cell survival on media containing spectinomycin.

Cells from 11-P[300] were transformed with the *rs-3* gene, which encodes a mutated form of *Chlamydomonas* protoporphyrinogen oxidase conferring resistance to diphenyl ether herbicides (41). Herbicide-resistant transformants were recovered and tested for their ability to grow on media containing different concentrations of spectinomycin. By using this approach, we isolated two mutant strains (Mut-9 and Mut-11) that showed reactivation of the chimeric *aadA* transgene (Fig. 1). In Mut-9 and Mut-11, the *rs-3* gene integrated into different genomic locations, providing a molecular tag to identify either mutant or a double mutant by Southern blot analysis (Fig. 1*A*). Blots of total cell DNA hybridized to the pBluescript vector backbone, common to plasmids containing the *aadA* or *rs-3* genes, showed a 4.5-kb *Hin*dIII fragment corresponding to the *RbcS2*::*aadA*::*RbcS2* transgene and ≈13 kb and ≈20 kb segments corresponding to the *rs-3* inserts in Mut-9 and Mut-11, respectively (Fig. 1*A*). Tetrad analyses confirmed that the introduced *rs-3* marker cosegregated with reactivation of expression of the *RbcS2*::*aadA*::*RbcS2* transgene (data not shown).

Expression of the *RbcS2*::*aadA*::*RbcS2* Transgene. We examined the expression of the chimeric *aadA* transgene by Northern blot analysis and by cell survival on medium containing 100 μg/ml of spectinomycin. Hybridization to the *aadA* coding sequence was observed in Mut-9, Mut-11, and in a double mutant, Mut-9 Mut-11, but was undetectable in the silenced strain 11-P[300] and in the untransformed wild-type strain CC-124 (Fig. 1*B*). As previously reported (11), the *RbcS2*::*aadA*::*RbcS2* transcripts showed several discrete bands superimposed on a smear of hybridizing RNA, presumably because of improper mRNA processing. As a control for equal loading of the lanes, the same

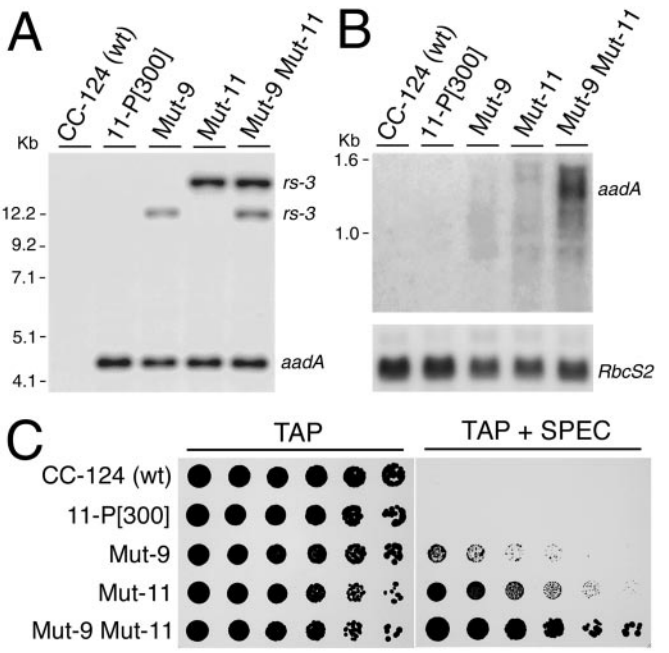

Fig. 1. Expression of the *RbcS2*::*aadA*::*RbcS2* transgene is reactivated in the mutant strains. (*A*) Southern blot analysis of the wild-type untransformed strain (CC-124), the silenced parental strain (11-P[300]), the mutant strains (Mut-9 and Mut-11), and a double mutant strain (Mut-9 Mut-11). Total cell DNA was digested with *Hin*dIII and hybridized to the pBluescript vector backbone, which is common to the plasmids containing the chimeric *aadA* transgene or the tagging *rs-3* gene. The fragments corresponding to the transgene (*aadA*) or the insertional mutagen (*rs-3*) are indicated. (*B*) Northern blot analysis of the strains described above. Total cell RNA was isolated from each strain, separated under denaturing conditions, and hybridized to the *aadA* coding sequence (*Upper*). The same blot was reprobed with the coding sequence of *RbcS2* as a control for equal loading of the lanes (*Lower*). The faint transcript seen above *RbcS2* corresponds to the *RbcS1* gene (11). (*C*) Growth and survival on TAP medium or on TAP medium containing spectinomycin (TAP + SPEC) of the indicated strains. Five-fold serial dilutions of cells, starting with 1×10^5 cells on the left, were spotted on each plate and incubated for 15 days (12).

blot was rehybridized with a probe specific for *RbcS2* (Fig. 1*B*). Consistent with the steady-state levels of *RbcS2*::*aadA*::*RbcS2* transcripts, Mut-9, Mut-11, and Mut-9 Mut-11 were able to grow in the presence of spectinomycin, whereas 11-P[300] and CC-124 could not survive (Fig. 1*C*). In addition, the double mutant showed greater *aadA* RNA levels (Fig. 1*B*) as well as greater resistance to spectinomycin (Fig. 1*C*) than either of the individual mutants. These results indicate that integration of the *rs-3* marker disrupted two genes, designated *Mut9* and *Mut11*, required for epigenetic silencing of the *RbcS2*::*aadA*::*RbcS2* transgene.

Reactivation of Transposable Elements. We also analyzed whether the mutations affected mobilization of *Chlamydomonas* transposons. Transposable elements are grouped into two major classes depending on their mode of transposition. Class I elements transpose via an RNA intermediate and include retrotransposons and other retroelements, such as *Chlamydomonas TOC1* (43). In contrast, Class II elements transpose via a DNA intermediate by a "cut-and-paste" mechanism and include *Chlamydomonas Gulliver* (44).

As previously reported (13, 43), the majority of *TOC1* transcripts are nonpolyadenylated and heterogeneous in size, which produces a smeary signal on Northern blots of total RNA (Fig. 2*A*). The steady-state level of *TOC1* RNA is about 2.5-fold higher in Mut-9 compared with the parental strain 11-P[300] (Fig. 2*A*). *TOC1* transcripts are also somewhat elevated in Mut-11. Accordingly, the transposition frequency of *TOC1* is significantly enhanced in Mut-9 but only slightly affected in Mut-11. Southern blot analyses of 10 parallel subcultures of 11-P[300], Mut-9, and Mut-11 revealed additional *TOC1* copies in the genome of many Mut-9 subclones (Fig. 2*B* and data not shown). In contrast, the Mut-11 subcultures displayed very few changes in the copies of *TOC1* (Fig. 2*B*, Mut-11 subclones 3 and 6), and the 11-P[300] subcultures showed no detectable transposition.

We also examined the mobilization of a Class II transposable element, *Gulliver* (Fig. 2*C*). Total cell DNA from 10 parallel subcultures of each strain was digested with *Hin*dIII and hybridized with a terminal repeat sequence of *Gulliver*. Whereas 11-P[300] and Mut-11 showed no changes in the banding pattern of *Gulliver*, a few subcultures of Mut-9 displayed differences indicative of transposon mobilization (Fig. 2*C*, Mut-9 subclones 1, 5, and 7). However, the changes were most dramatic in the double mutant Mut-9 Mut-11, where many subcultures showed missing fragments (indicating excision from the genome) as well as new fragments (indicating integration into other genomic locations) (Fig. 2*C*). These observations suggest that *Mut9* and *Mut11*, in addition to their role in the epigenetic silencing of transgenes, participate in the suppression of transposable elements.

Photoheterotrophic Cell Growth. Because Mut-9 and Mut-11 were deficient in both transgene and transposon silencing, we tested for defects in other biological processes that might indicate additional roles of the mutated gene products on global gene regulation. To determine growth rates, cells pregrown to logarithmic phase were inoculated at low density into fresh medium and cultured under the same conditions. Cell densities were measured at fixed intervals. The growth rate of Mut-9 was similar to that of the wild-type strain CC-124 under standard photoheterotrophic conditions (Fig. 3). In contrast, Mut-11 and the double mutant grew at a slower rate. In the exponential phase of growth, all mutants had doubling times similar to that of the wild-type strain (Fig. 3). However, Mut-11 and the double mutant showed a much longer lag phase. Thus, Mut-11 seems to be defective in the initial survival and/or adaptation to grow at low density in new medium, suggesting that *Mut11* might regulate a physiological adaptive response(s).

Sensitivity to DNA-Damaging Agents. Because of the possible connections between DNA repair and chromosomal mechanisms of epigenetic regulation (38, 39, 45–50), we also examined the response of the mutants to several genotoxic agents. Mut-9 and Mut-11 were particularly sensitive to chemical agents that induce DSBs (51), such as methyl methanesulfonate or bleomycin (Fig. 4 *A* and *C*). The dose resulting in 30% cell survival (Fig. 4, horizontal dashed lines) was significantly lower for each mutant compared with the wild-type strain CC-124. Moreover, the double mutant was much more sensitive to these treatments than each of the single mutants. In contrast, Mut-11 was as resistant as the wild-type strain to UV-C irradiation (<280 nm) (Fig. 4*B*), a treatment that mainly causes formation of cyclobutane pyrimidine dimers (51). Similarly, Mut-9 and Mut-9 Mut-11 showed only a moderate defect in survival on exposure to low doses of UV-C light, although they were clearly sensitive at higher doses. Mut-9 also displayed a greater than 10-fold reduction in the frequency of transformation with exogenous DNA, when compared with the parental strain 11-P[300] (see Table 1, which is published as supporting information on the PNAS web site, www.pnas.org). These observations are consistent with a deficiency in the integration of transforming DNA into the nuclear genome, presumably because of defective DSB repair.

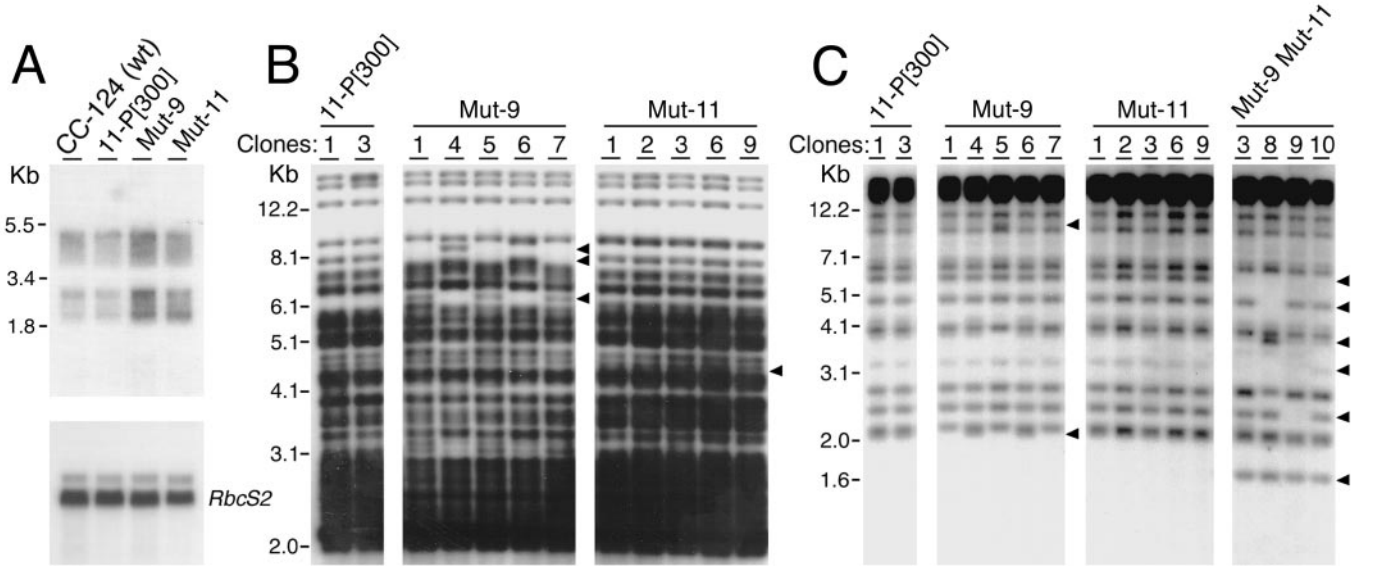

Fig. 2. Reactivation of a retroelement, *TOC1*, and a DNA transposon, *Gulliver*, in the mutant strains. Abbreviations are as in the legend to Fig. 1. (*A*) Northern blot of total RNA probed sequentially for *TOC1* (*Upper*) to examine transcript levels and for *RbcS2* (*Lower*) to test for equal loading of the lanes. (*B*) Southern blot analysis of *TOC1* transposition. Genomic DNA from parallel subcultures (Clones) of the indicated strains was digested with *Hin*cII and probed for *TOC1*. The arrowheads indicate new fragments in the subclones of Mut-9 and Mut-11. (*C*) Southern blot analysis of *Gulliver* transposition. Total cell DNA from parallel subcultures (Clones) of the indicated strains was digested with *Hin*dIII and probed for *Gulliver*. The arrowheads indicate missing or new fragments in the subclones of Mut-9 and Mut-9 Mut-11. Although only two subclones are shown for 11-P[300], we did not detect mobilization of either *TOC1* or *Gulliver* in 10 parallel subcultures grown under the same conditions as the mutant strains.

Discussion

Epigenetic Silencing of Transgenes. In *C. reinhardtii*, nuclear run-on assays with isolated nuclei and Northern blot analyses have revealed that transgene inactivation occurs at both transcriptional and posttranscriptional levels (12, 13). We describe here the characterization of two *Chlamydomonas* mutants, Mut-9 and Mut-11, defective in the epigenetic silencing of transgenes. The strain used to isolate these suppressors, 11-P[300], contains a single copy of the *RbcS2*::*aadA*::*RbcS2* transgene that is silenced at the transcriptional level without detectable cytosine methylation (12). Mut-9 and Mut-11 reactivate expression of this chimeric *aadA* transgene, as shown by Northern blot analyses and by the ability of the mutant cells to survive on spectinomycin-containing medium. Moreover, a double mutant (Mut-9 Mut-11) exhibited more pronounced transgene reactivation than each of the single mutants. Nuclear run-on assays confirmed that the *RbcS2*::*aadA*::*RbcS2* transgene becomes transcriptionally active in the mutant backgrounds (data not shown). Thus, *Mut9* and *Mut11* are required for the transcriptional silencing of transgenes in *Chlamydomonas*.

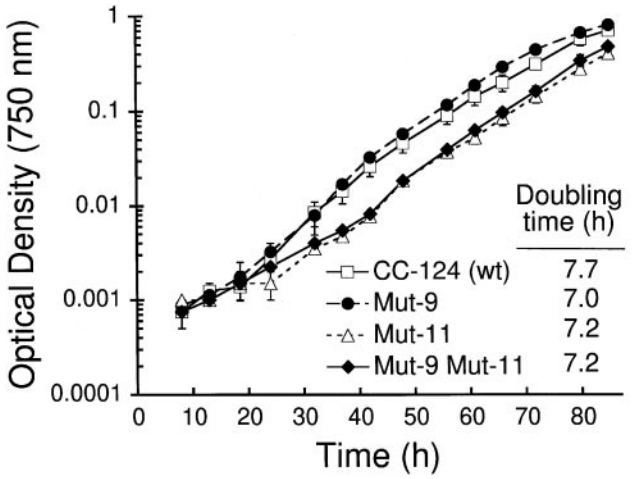

Fig. 3. Photoheterotrophic growth of the mutant and wild-type strains. Abbreviations are as in the legend to Fig. 1. Each time point represents the mean (± standard error) of six replicates (three independent experiments). Where the error bars are not visible, they are smaller than the symbols. The exponential phase of the growth curve was used to calculate doubling times. Even though all strains show similar doubling times, Mut-11 and Mut-9 Mut-11 took considerably longer to reach exponential growth (represented by a linear increase in optical density in the semilogarithmic scale).

Epigenetic Silencing of Transposons. Transposable elements are widespread constituents of all eukaryotic genomes (52). Epigenetic processes, particularly DNA methylation, have been implicated in regulating the activity of plant transposable elements (53). In *Arabidopsis*, Robertson's *Mutator* transposons and members of the CACTA superfamily are controlled by the SWI2/SNF2 chromatin-remodeling gene *DDM1* (28, 29). *DDM1* also has a slight effect on endogenous retrotransposon mobilization (27, 54). A chromomethylase (encoded by *CMT3*), which is required for maintenance of CpXpG methylation, has also been shown to participate in the silencing of retrotransposons (30). Moreover, a truncated *Athila* (a putative retrotransposon) transcript was induced in several *Arabidopsis* mutants defective in TGS (27).

The effect(s) of posttranscriptional gene silencing mechanisms on transposon mobilization in plants has not been reported. However, in *C. elegans* and *D. melanogaster*, transposon and/or retrotransposon mobilization is regulated by RNA interference/PTGS processes (8, 9, 55, 56). Similarly, we have previously reported that a *Chlamydomonas* mutant defective in PTGS shows enhanced transpositional activity of both *TOC1* and *Gulliver* (13). We have now found that mutations affecting TGS also enable mobilization of transposable elements in *Chlamydomonas*. The steady-state RNA level of the *TOC1* retrotransposon, as well as its transposition frequency, is enhanced in Mut-9

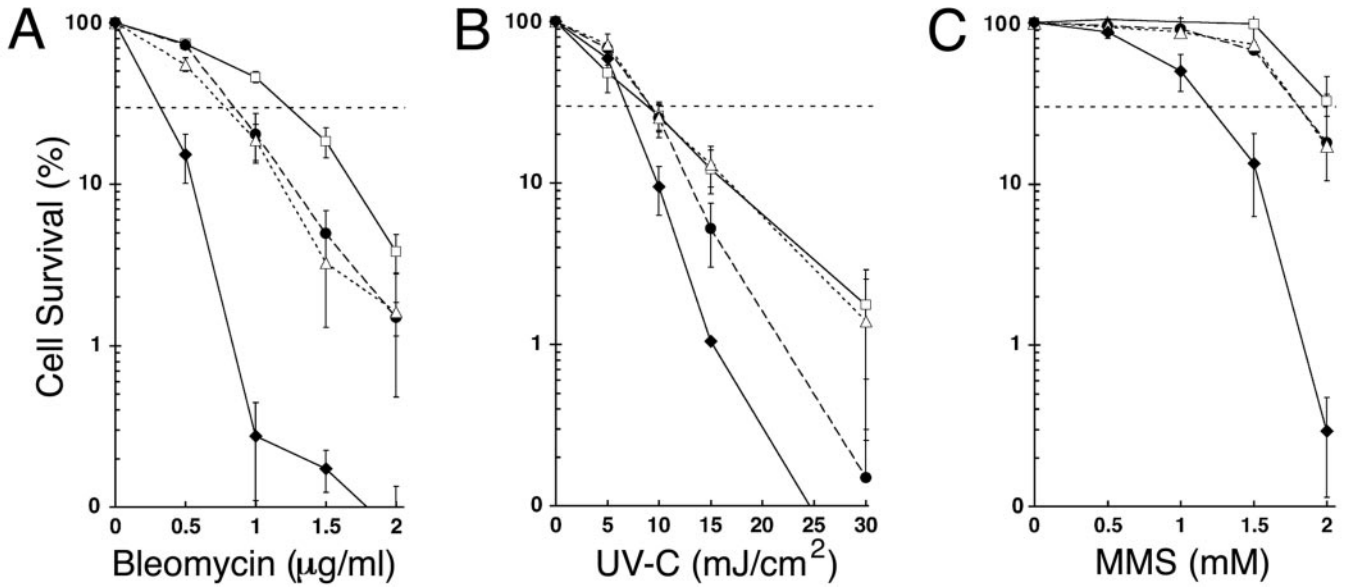

Fig. 4. Effect of DNA-damaging agents on the survival of the mutant and wild-type strains. Each graph point represents the mean (± standard error) of nine replicates (three independent experiments). Where the error bars are not visible, they are smaller than the symbols. The dashed horizontal lines indicate 30% cell survival. Symbols: □, wild-type CC-124; ●, Mut-9; Δ, Mut-11; ◆, Mut-9 Mut-11. (*A*) Survival of the mutants and wild-type *C. reinhardtii* grown on TAP medium containing increasing concentrations of bleomycin. (*B*) Survival of the mutants and wild-type *C. reinhardtii* exposed to increasing doses of UV-C irradiation under nonphotoreactivating conditions. (*C*) Survival of the mutants and wild-type *C. reinhardtii* grown on TAP medium containing increasing concentrations of methyl methanesulfonate (MMS).

compared with the parental strain 11-P[300]. The transpositional activity of the DNA element *Gulliver* is also slightly increased in Mut-9. Mut-11 shows some mobilization of *TOC1*, but the activity of *Gulliver* is not affected. Interestingly, in the double mutant, *Gulliver* seems to transpose at a much higher frequency than in either of the single mutants. Taken together, our results suggest that *Chlamydomonas* transposable elements are regulated by multiple epigenetic mechanisms operating at both transcriptional and posttranscriptional levels. Furthermore, there is also redundant repression at the transcriptional level because, as discussed below, *Mut9* and *Mut11* appear to play a role(s) in partly different pathways. Likewise, the *I* factor retrotransposon in *D. melanogaster* appears to be controlled by various epigenetic processes (55, 57). Thus, multiple epigenetic mechanisms might operate as a defense system against the massive expansion of transposable elements in eukaryotes.

Molecular Mechanism(s) of Epigenetic Transcriptional Silencing. Molecular characterization of the suppressor strains provided insights into the silencing mechanism(s). Because of deletions caused by integration of the *rs-3* plasmid, the mutations in Mut-9 and Mut-11 result in complete loss-of-function null phenotypes (data not shown). Since a double mutant shows greater transgenic reactivation and greater transposon mobilization than either of the single mutants, epistatic analysis suggests that *Mut9* and *Mut11* function in (at least) partly distinct pathways of transcriptional repression. This explanation is also supported by the differences in the phenotypes of Mut-9 and Mut-11.

Phenotypic characterization of the parental strain 11-P[300] suggested the involvement of chromatin domains in transcriptional silencing of unmethylated transgenes (12). Consistent with this interpretation, the *Mut9* and *Mut11* gene products (Mut9p and Mut11p) might function in the formation of a repressive chromatin structure that leads to the transcriptional inactivation of transgenes and transposons. *Mut11* encodes a WD40-repeat containing protein (GenBank accession no. AF443204) with homology to the C-terminal domain of *Saccharomyces cerevisiae* Tup1p, a global transcriptional repressor (58–60). Yeast Tup1p interacts with many proteins, including components of the basal transcription machinery, histones, and histone deacetylases, and has been suggested to play an architectural role in organizing repressive chromatin domains (58–60). By analogy, Mut11p may also have a structural function and/or interfere directly with transcription factors. *Mut9* encodes a novel serine/threonine protein kinase (GenBank accession no. AF443205). In *D. melanogaster*, phosphorylation of Heterochromatin Protein 1 is correlated with heterochromatin assembly and silencing (61). Perhaps in similar fashion, Mut9p may modulate chromatin structure by phosphorylation of one or more of its components.

Transcriptional Silencing, DNA Repair, and Cell Growth. Although Mut-9 and Mut-11 were isolated on the basis of their ability to reactivate expression of the *aadA* transgene, they also show enhanced sensitivity to DNA-damaging agents, particularly radiomimetic chemicals inducing DSBs. In addition, Mut-9 displays a greater than 10-fold reduction in the frequency of transformation with foreign DNA, presumably because of a deficiency in the integration of transforming DNA into the nuclear genome. As discussed below, these results are consistent with a role of *Mut9* and *Mut11* in the repair of DSBs. It should be noted, however, that increased sensitivity to DNA-damaging agents could also result from overall defects in genome stability and cell survival. However, this explanation seems less likely because the mutants are only moderately sensitive to UV-C irradiation, and Mut-9 is not affected in cellular growth.

DSBs are a common form of DNA damage in proliferating cells, and their repair is a fundamental mechanism of genome protection (62). Observations in a variety of eukaryotic organisms suggest that the repair of DSBs is associated with chromatin modifications (39). After exposure to ionizing radiation, a member of the histone H2A family becomes rapidly phosphorylated in domains around the damaged sites (38, 39). In *S. cerevisiae*, DSBs cause the relocalization of *SIR* (Silent Information Regulator) proteins from telomeres, where they are responsible for epigenetic silencing, to the site of damage (45, 46). The *ATM* (Ataxia Telangiectasia Mutated) and *ATR* (*ATM*

and *Rad3* Related) protein kinases, which have been implicated in the response of mammalian cells to multiple forms of DNA damage, are present in complexes with histone deacetylases and/or chromatin remodeling factors (47, 48). The ATM-associated deacetylase activity increases on cellular exposure to ionizing radiation (47). Moreover, factors involved in DNA repair, DNA replication, and chromatin assembly, such as CAF-1 (Chromatin Assembly Factor 1) and PCNA (Proliferating Cell Nuclear Antigen), have been implicated in a marking system for the inheritance of epigenetic states (49, 50). Even though these processes have not been examined in detail in plants, it is intriguing that mutagenesis treatments, such as exposure to ethyl methanesulfonate or irradiation, occasionally lead to the formation of silenced epi-alleles in genes regulating plant development (3, 63). The latter observations and our results suggest that the connections between DNA repair and epigenetic chromosomal mechanisms may also extend to the plant kingdom.

In *Chlamydomonas*, Mut9p and Mut11p might play a role in establishing the proper chromatin environment for DNA repair. Because Mut9p is a protein kinase, it might also participate in the signaling response to DNA damage. Indeed, many proteins directly involved in DSB repair or cell-cycle checkpoints are regulated by phosphorylation (62, 64). Another explanation for the mutant phenotypes is that Mut9p and Mut11p might control the expression of genes required for DNA repair, perhaps indirectly, as reported in yeast for SIR regulation of the non-homologous end-joining repair pathway through mating type factors (65).

Besides sensitivity to DNA-damaging agents, Mut-11 also shows defects in growth when cells are inoculated into fresh medium at low density. *S. cerevisiae* Tup1p is required for the repression of multiple families of genes, including those responsive to different physiological conditions such as osmotic stress and hypoxia (58, 60). By analogy, Mut11p might participate in the regulation of genes involved in a physiological adaptive response(s).

In summary, *Chlamydomonas* Mut-9 and Mut-11 are defective in the transcriptional silencing of transgenes, the repression of transposable elements, the tolerance of DNA damage (particularly DSBs), and, in the case of Mut-11, cell growth. The simplest explanation for these pleiotropic phenotypes is that Mut9p and Mut11p are involved in the formation of a distinct chromatin structure that is required, directly or indirectly, for repression of transgenes and transposons, for controlling endogenous gene expression, and possibly for repairing DNA damage.

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